REMARKS

I. Preliminary Remarks and Amendments

Claims 1-10 are currently pending. Claims 1-7 and 9-10 are under examination and were variously rejected under 35 U.S.C.§112, first paragraph, for lack of enablement; §112, second paragraph; and §102(b) over either Orlic et al. (*P.N.A.S.* 98:10344-10349, 2001) or Anversa (Pre-grant Patent Publication No. US 2002/0061587 A1 [05/2002]). Claims 1 and 5 are amended herein. Support for the amendment to claim 1 is found throughout the specification, including page 20, lines 14-17. Support for the amendment to claim 5 is found throughout the specification, including page 4, line 30, through page 5, line 2; and page 11, lines 14-30. Accordingly, the amendments do not include new matter. The Applicants do not intend with these or any other amendments to abandon the subject matter of claims previously presented, and reserve the right to pursue such subject matter in duly filed continuing patent applications.

II. Patentability Arguments

A. The Rejections Under 35 U.S.C. §102(b) May Properly Be Withdrawn.

1. Orlic does not anticipate the subject matter of any pending claim.

The Examiner rejected claims 1, 2, and 9 under 35 U.S.C. §102(b) for anticipation by Orlic et al., *Proc. Natl. Acad. Sci.* 98:10344-10349, 2001 (hereinafter "Orlic"), because the Examiner asserted that Orlic assertedly discloses that the use of G-CSF and SCF in conjunction can positively influence myocardial outcomes following ischemia and reperfusion, such as improved wall thickness and increased ejection fraction when compared to untreated animals. *See* Office Action at page 5. In response, the Applicants submit that Orlic does not disclose each limitation of any one of the rejected claims, as amended.

Orlic showed that the mobilization of primitive bone marrow cells **prior to acute myocardial infarction (AMI)**, by the prophylactic administration (prior to the ischemic event) of SCF in combination with G-CSF, resulted in a significant degree of tissue regeneration in the ischemic site. The present invention offers an effective treatment for AMI or arterial occlusion **after** the ischemic event has already occurred. In addition, Orlic

administered SCF in conjunction with G-CSF and does not disclose the use of G-CSF alone in the treatment of AMI. Moreover, Orlic attributed most of the success of their method to SCF. For example, Orlic theorized that "SCF could be responsible for migration, accumulation, and multiplication of primitive BMC in the infarcted zone. . ." *See* Orlic at p. 10349, column 1, paragraph 2. Orlic also does not disclose the use of G-CSF alone in the treatment of an occlusion in an artery. Orlic administered SCF in conjunction with G-CSF prophylactically in the treatment of AMI. Orlic did not demonstrate nor contemplate that treatment with G-CSF alone **after** an arterial occlusion could have any beneficial effect. In summary, Orlic administered SCF and G-CSF prior to inducing AMI, and then observed the effect that this protocol had on tissue regeneration in the heart. Orlic did not treat with G-CSF after AMI or after an ischemic event and, therefore, does not anticipate claims 1 or 9.

As a matter of law, a dependent claim incorporates each limitation of a claim from which it depends. 35 U.S.C. §112, fourth paragraph. Claim 2 depends from claim 1 and, as established above, Orlic does not disclose each element of claim 1, as amended. Accordingly, Orlic cannot disclose, expressly or inherently, each limitation of dependent claim 2 and, for that reason, Orlic does not anticipate the subject matter of any dependent claim.

For the foregoing reasons, Orlic does not anticipate the subject matter of any of claims 1, 2, and 9 under 35 U.S.C. §102(b) and, therefore, the rejection should be withdrawn.

2. Anversa does not anticipate the subject matter of any pending claim.

The Examiner rejected claims 1-7 and 9-10 under 35 U.S.C. §102(b) for anticipation by Anversa, (Pre-grant Patent Publication No. US 2002/0061587 A1 [05/2002]; hereinafter "Anversa"), because Anversa assertedly discloses that it is well known in the art that reperfusion therapy focuses on re-establishing blood flow through such methods as angioplasty, thrombolysis, and coronary bypass; however, these methods have no effect on irreversibly damaged tissue. *See* Office Action at page 6. The Examiner also asserted that Anversa discloses a method of reperfusion therapy comprising administering cytokines, including G-CSF, SCF, GM-CSF, IL-3, etc. Anversa's method and compositions assertedly induce stem cell mobilization and migration, which aids in the regenerative process of the

heart following ischemia and reperfusion. Anversa's method also assertedly showed protection against ischemia and reperfusion as evidenced by improved wall thickness. The Examiner also asserted that Anversa's method assertedly discloses particular doses from 50 μg/kg to 500 mg/kg and can be used on any vertebrate, including humans. In response, the Applicants submit that Anversa does not disclose each limitation of any one of the rejected claims, as amended.

Anversa does not disclose the use of G-CSF alone in a method of reperfusion therapy for the treatment of AMI as is claimed in the present invention. Anversa's method comprises the delivery of somatic stem cells, alone or in combination, with cytokines, including SCF, G-CSF, GM-CSF, IL-3, etc. (*see* Anversa at p. 1, paragraph [005]). Anversa does not teach in paragraph [005], as the Examiner asserted, a method of reperfusion therapy. Instead, Anversa's paragraph [005] discloses that the methods and/or pharmaceutical compositions of his invention comprise an effective amount of stem cells, alone or in combination with, a cytokine. In fact, none of Anversa's methods involve the treatment of mammals with G-CSF in conjunction with reperfusion therapy after myocardial infarction to reduce heart damage (*see* Anversa's Examples 1-7; paragraphs [0160-0201]. Anversa's Example 2, for example, uses only the prophylactic treatment of SCF in combination with G-CSF for five days prior to an induced AMI to improve survival, promote myocardial regeneration, reduce infarct size, and increase posterior wall thickness (same results as published by Orlic as discussed above). Anversa did not disclose the use of G-CSF in a reperfusion therapy method for improved patient outcome or increased ventricular wall thickness.

Although Anversa's methods and compositions may assertedly induce stem cell mobilization and migration and aid in the regenerative process of the heart following ischemia and reperfusion, Anversa does not anticipate the present invention. Anversa showed that the mobilization of primitive bone marrow cells **prior to AMI**, by the prophylactic administration of SCF in combination with G-CSF (prior to the ischemic event), resulted in a significant degree of tissue regeneration in the ischemic site. Anversa also does not disclose the use of G-CSF alone in the treatment of an occlusion in an artery. Anversa did not demonstrate nor contemplate that treatment with G-CSF alone **after** an arterial occlusion could have any beneficial effect. Thus, Anversa does not anticipate claims 1 or 9.

As set out above, a dependent claim incorporates each limitation of a claim from which it depends. Thus, claims 2-7 depend from claim 1 and claim 10 depends from claim 9 and, as established above, Anversa does not disclose each element of claims 1 or 9, as amended. Accordingly, Anversa cannot disclose, expressly or inherently, each limitation of any of dependent claims 2-7 and 10 and, for that reason, Anversa does not anticipate the subject matter of any dependent claim.

For the foregoing reasons, Anversa does not anticipate the subject matter of any of claims 1-7 and 9-10 under 35 U.S.C. §102(b) and, therefore, the rejection should be withdrawn.

B. The Rejections Under 35 U.S.C. §112, First Paragraph, May Properly Be Withdrawn.

The Examiner rejected claims 1 and 5 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for a method of improving wall thickness following ischemia and reperfusion, does not assertedly provide enablement for the broad claim of a method of reducing all forms of heart damage following ischemia and reperfusion. In response, the Applicants respectfully traverse the rejection.

The question for enablement of such subject matter is whether one of skill in the art having been taught the method of claim 1 can determine various forms of reduction in heart damage without undue experimentation. Enablement is not precluded by the necessity for some experimentation; indeed it is inevitable that there may be some quantity of experimentation required. The mere fact that some degree of experimentation may be required is not the determinative factor in the scope of enablement; it is only when the level of experimentation becomes undue that it is fatal to the enablement of an invention. Thus, the key word is undue, not experimentation. *In re Wands* 858, F.2d 731, 8 USPQ 2d 1400, 1404 (Fed. Cir. 1988). A determination of what constitutes undue experimentation in a case requires application of a test of reasonableness giving regard to the nature of the invention and the state of the art. *Id.* The test is not merely quantitative since a considerable amount of experimentation is permissible if it is merely routine or if the specification provides a reasonable amount of guidance. *Id.* Applying the standard articulated in *In re Wands*, the present specification provides a reasonable amount of guidance to one of skill in the art to determine reduction in heart damage. The specification indicates that one skilled in the art

would look for reductions in heart damage by looking for 1) induced neoangiogenesis in the infarcted zone, 2) reduced cardiomyocyte apoptosis, 3) reduced necrosis, 4) reduced scar formation, 5) improved cardiac function, 6) decreased infarct-related myocardial thinning, and 7) improved ventricular function. *See* specification at page 6, lines 13-19; page 9, lines 4-11; page 19, lines 9-13; and page 20, lines 31-32. Thus, the invention is objectively enabled and nothing more is required to satisfy the first paragraph of §112. *In re Marzocchi*, 169 USPQ 367,369 (CCPA 1971).

Furthermore, it is a well known tenet of the law that a specification disclosure need not teach, and preferably should omit, what is well known to those of skill in the art. *In re Buchner*, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991). As long as the specification contains at least one method of making and using the claimed invention that bears a reasonable correlation to the entire scope of the claimed invention, then the enablement requirement under 35 U.S.C. §112 is satisfied. *In re Fisher*, 166 USPQ 18, 24 (CCPA, 1970); MPEP 2164.01(b). Example 1 provides such a method of using the claimed invention, and given the level of skill in the art the skilled artisan could perform the methods of the invention and identify other types of reduction in heart damage. Such an exercise would be mere routine experimentation for one skilled in the art. There is no requirement for specific working examples in the specification for all that is claimed in the invention. Moreover, there can be no doubt that in the year 2004, contemporaneously with the filing date of the present application, one of skill in the art could determine the effect of treatment on various measures of heart damage using the methods of the invention and using nothing more than routine experimentation.

The Examiner also rejected claim 1 because the range for "an effective amount" of a composition comprising G-CSF polypeptide assertedly would never be considered an acceptable range when dealing with compositions and the use of compositions in animal, and especially human, procedures. Therefore, it would not be possible to use the invention without experiments to support the broad range of concentrations in an "effective amount" due to the quantity of experimentation necessary, the lack of an adequate number and representative working examples, the nature of the invention, and the breadth of claims. The Applicants respectfully traverse this rejection.

The specification provides a range of dosages and teaches that a preferable dose would be approximately 300 μg per day. The specification also provides a working

example that demonstrates that a dosage of 300 μg administered every other day in a porcine model of male Yucatan mini-pigs (which normally weigh between 25-35 kg) was effective in treating acute AMI in a mammal to reduce heart damage by improving wall thickness in the infarct zone (*see* Example 1, pages 20-21). Furthermore, G-CSF has been administered clinically for many years and one of skill in the art is familiar with appropriate dosages for this compound. For example, Neupogen® (Amgen Inc.) is administered in human patients after bone marrow transplant at a dosage of 10 μg /kg/day. Thus, the effective dosage for a porcine model exhibited in the present invention is almost identical to that recommended for human patients after bone marrow transplant. In addition, others skilled in the art (*see* Orlic and Anversa as cited above by the Examiner) have used 50 μg/kg/day in the pretreatment of a rat model of AMI. Therefore, the Applicants submit that one of skill in the art would be able to determine, without undue experimentation, an appropriate dosage, i.e. an "effective amount," of G-CSF for the treatment of AMI or arterial occlusion in humans and other mammals.

Finally, the Examiner also rejected claim 5 for asserted lack of enablement because claim 5 is drawn to a method of improved reperfusion therapy using a composition comprising G-CSF and numerous cytokines that are proinflammatory, such as IL-8. The Examiner asserted that IL-8 is an inflammatory cytokine that is upregulated during ischemia and reperfusion, including coronary artery bypass (Vallely et al., *J. Thorac. Cardiovasc. Surg.* 124:758-767, 2002, esp. Figure 1; hereinafter "Vallely"), and, therefore, this cytokine would actually increase heart damage by increasing inflammation. The Examiner also moved that none of the cytokines recited in claim 5 were ever combined with G-CSF in the examples to show the claimed method would in fact display any protection against ischemia and reperfusion. The Examiner thus questioned whether the invention could be practiced with any degree of success, and concluded that it would not be possible to make/and or use the invention commensurate in scope due to the quantity of experimentation necessary, the absence of an adequate number of working examples, the nature of the invention, the state of the prior art, and the predictability of the art. The Applicants respectfully traverse this rejection.

Although the Examiner has suggested that Vallely indicates that IL-8 is pro-inflammatory and suggests that this cytokine would increase heart damage, there have been other reports, e.g., Laterveer et al. (*Exp. Hematol.* 24:1387-1393, 1996; enclosed herewith as **Appendix A**), which have shown that IL-8 induces instant mobilization of hematopoietic

progenitor cells in mice and primates. The use of IL-8 for the mobilization of such progenitor cells may be helpful in assisting in the repair or prevention of heart damage after an AMI. Also, one of skill in the art is aware that growth factors may have many different functions depending on the circumstances. Therefore, the Applicants submit that one of skill in the art might use IL-8 in conjunction with G-CSF in the methods of the invention.

Likewise, the Examiner has no basis for rejecting the Applicant's contention that the use of at least one additional cytokine, recited in claim 5, will have a beneficial effect on outcome when combined with G-CSF in the treatment of ischemia with reperfusion. As set out above, there is no requirement for specific working examples in the specification. Furthermore, each of these cytokines has been shown to have a role in stimulating growth and proliferation of hematopoietic cells, which may be useful in combination with G-CSF in reducing damage to the infarcted area of the heart. There can be no doubt that one of skill in the art could easily determine the effect of treatment with one of these cytokines in combination with G-CSF on heart damage using the methods of the invention and using nothing more than routine experimentation.

In view of the above discussion, Applicants respectfully request that the rejection of claims 1 and 5 for lack of enablement be withdrawn.

C. The Rejection Under 35 U.S.C. § 112, Second Paragraph, May Properly Be Withdrawn.

The Examiner rejected claim 5 under 35 U.S.C. §112, second paragraph, because the phrase "includes the use of at least" assertedly renders the claim indefinite because it is unclear whether the limitation(s) following the phrase are part of an independent step or if the limitations following the phrase are supposed to be co-administered simultaneously with G-CSF. In response, the Applicants respectfully traverse the rejection.

The specification provides that G-CSF may be administered in a single composition with another agent or in two distinct compositions (*see* specification at page 12, lines 3-7). Thus, the Applicants submit that claim 5, as originally filed, is not indefinite and is fully supported by the specification. Nevertheless, in order to expedite prosecution, the Applicants have amended claim 5 to replace the assertedly indefinite phrase "includes the use of" with "comprises at least. . ." Support for this amendment is found throughout the specification and at page 4, line 30, through page 5, line 2; and page 11, lines 14-30.

In view of the amendment to claim 5, the Applicants respectfully submit that the rejection of claim 5 for indefiniteness has been overcome and should be withdrawn.

III. Conclusion

In view of the amendments and remarks made herein, the Applicants respectfully submit that claims 1-7 and 9-10 are in condition for allowance and respectfully request expedited notification of same. Should the Examiner have any questions, he is welcomed to contact the undersigned at the telephone number below.

Respectfully submitted,

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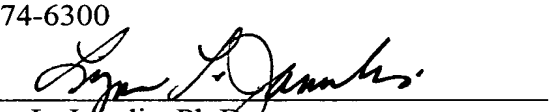
By: 

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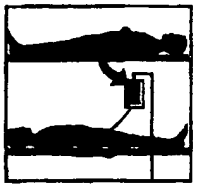
Experimental Hematology 24:1387–1393 (1996)

Experimental Hematology



Improved survival of lethally irradiated recipient mice transplanted with circulating progenitor cells mobilized by IL-8 after pretreatment with stem cell factor

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Abstract

We have demonstrated previously that a single bolus-injection of interleukin (IL)-8 induces instant mobilization of hematopoietic progenitor cells (HPC) in mice and primates. To further improve the mobilization of HPC, we treated mice with hematopoietic growth factors (HGF) before IL-8-administration. The mobilized HPC were transplanted into lethally irradiated recipient mice to study the effects on survival. Male donor mice (age 8–12 weeks, weight 20–25 grams) were pretreated intraperitoneally (ip) with a fixed dose of 2.5 μg of either granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, stem cell factor (SCF), or saline administered twice daily for 2 to 4 days. Then a fixed dose of 30 μg of IL-8 was administered ip at various time intervals before harvesting blood, bone marrow, and spleen. Cell counts and numbers of colony-forming units granulocyte/macrophage (CFU-GM) of these organs were assessed. Donor mice pretreated with HGF for 2 days and subsequently injected with IL-8 showed an increase in the numbers of circulating CFU-GM per mL blood from 168 ± 98 to 402 ± 201 (mean ± SD, CFU-GM/mL blood) when GM-CSF was used, 314 ± 133 to 2502 ± 513 with G-CSF, and 27 ± 15 to 524 ± 339 with SCF compared with saline-pretreated controls (28 ± 17 to 462 ± 335 CFU-GM/mL blood, mean ± SD; n=42 and 40 per interval). Donor-mice pretreated for 4 days with IL-3 or GM-CSF showed an increase in the numbers of circulating HPC from 62 ± 52 to 368 ± 118 and 859 ± 387 to 1034 ± 421, respectively (CFU-GM/mL, mean ± SD, n=4 per group). Lethally irradiated (8.5 Gy) female Balb/c mice were then injected with decreasing numbers of peripheral blood mononuclear cells (PBMNC). Transplantation of 1.5×10^5 MNC obtained from donors pretreated with SCF for 2 days prior to IL-8 mobilization resulted in a significantly enhanced survival of 100% of the recipients, whereas recipients of PBMNCs derived from donors treated with SCF only or IL-8 as a single injection had a survival rate at day 60 of only 50% and 60% respectively. When equal numbers of IL-8-mobilized MNCs from G-CSF, GM-CSF, or IL-3 pretreated donors were transplanted into lethally irradiated recipients, no such survival-advantage was observed. We conclude that pretreatment with SCF for 2 days improves the mobilizing effect induced by IL-8 and that transplantation of these cells enhances survival of lethally irradiated recipients.

Key words: Stem cell transplantation—Hematopoietic progenitor cells—Stem cell factor—Stem cell mobilization—Radiation

Introduction

IL-8 is a member of the CXC-chemokine family and is predominantly involved in the activation and migration of neutrophils. IL-8 is produced by a variety of cells, i.e., monocytes, neutrophils, fibroblasts, endothelial cells, lung epithelial cells, mast cells, and keratinocytes [1–13] in response to stimulation with lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), GM-CSF, IL-1, IL-2, or IL-3 [14–17]. In vitro, IL-8 activates neutrophils and stimulates chemotaxis, induces the release of storage enzymes and the production of toxic metabolites in neutrophils, inhibits neutrophil-endothelial interaction, upregulates CD11b/CD18, and induces shedding of L-selectin and trans-endothelial migration of neutrophils [1–5,18–24].

Local injection of IL-8 in vivo induces granulocytosis, neutrophil margination and infiltration, plasma exudation, and angiogenesis in monkeys, rabbits, rats, and mice [5,25–32]. Systemic administration results in an instant neutropenia followed by neutrophilia lasting for several hours [25]. The mobilized neutrophils include both mature and immature cells, showing mobilization of cells out of the bone marrow reserve [30,33].

Previously we showed that a single injection of IL-8 is capable of mobilizing hematopoietic progenitor cells in a rapid and reproducible fashion in mice and nonhuman primates [32,33]. In mice and rhesus monkeys, maximal numbers of circulating progenitor cells are reached at 15 to 30 minutes after a single bolus injection of IL-8. These cells are capable of protecting lethally irradiated mice and exhibit long-term repopulating ability in cells of the myeloid as well as the lymphoid lineages. Thus, IL-8 induces mobilization of true hematopoietic stem cells.

Reports have been published indicating that treatment with some combinations of growth factors are superior to single factors in stem cell mobilization [34–39]. To investigate whether hematopoietic growth factors were able to enhance the IL-8-induced stem cell-mobilization, we studied the effects of pretreatment of donor mice with various hematopoietic growth factors prior to mobilization of hematopoietic progenitor cells by IL-8. We show that trans-

plantation of PBMNCs derived from donor mice pretreated with SCF prior to mobilization with IL-8 results in enhanced survival of lethally irradiated (8.5 Gy) recipients compared with recipients of PBMNCs derived from animals treated with either SCF or IL-8 alone.

Material and methods

Mice

Male and female Balb/c mice with an age ranging between 8–12 weeks were purchased from Broekman BV (Someren, The Netherlands). Animals were fed commercial rodent chow and acidified water ad libitum. They were maintained in a pathogen-free environment and were fed water containing ciprofloxacin 1 mg/mL (Bayer Nederland BV, Mijdrecht, The Netherlands), polymyxin-B 70 µg/mL and saccharose 2 g/100 mL. In some experiments, splenectomized mice were used [40]. In transplantation experiments, recipient female mice were placed in a polymethylmeta-acetate (PMMA) box and given total body irradiation (8.5 Gy, Philips SL 75-5/6 mV linear accelerator, Philips Medical Systems, Best, The Netherlands), divided in two parts in posterior-anterior and anterior-posterior position, at a dose rate of 4 Gy/minute. Male blood-derived MNCs were injected in the tail vein of lethally irradiated female recipients.

Cytokines

Human recombinant *E. Coli*-derived IL-8 [18] was obtained from the laboratory of I.J.D.L. (Sandoz Forschungsinstitut, Vienna, Austria). IL-8 had no colony-stimulating activity as reported previously [41]. The concentration of endotoxin was less the 0.05 EU/mL as tested in the Limulus amoebocyte lysate assay. Recombinant murine GM-CSF, IL-3, and SCF were provided by the Biotechnology Unit of Sandoz (Basel, Switzerland). Recombinant human G-CSF and SCF were provided by Amgen (Thousand Oaks, CA). For in vivo experiments, all agents were diluted to the desired concentration in endotoxin-free phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and administered as an ip injection. Prior to IL-8-induced mobilization, growth factors were administered at fixed doses of 2.5 µg/mouse twice daily for 2 or 4 days. In some experiments, G-CSF and GM-CSF (2.5 µg/mouse) were administered as a single ip injection.

Preparation of cell suspensions

Mice were killed by CO_2 asphyxia at various time intervals after IL-8 injection. Peripheral blood was drawn by cardiac puncture, and white blood cell counts were performed on a Sysmex® F800 (Toa Medical Electronics, Kobe, Japan). Neutrophil counts were performed on May Grunwald-Giemsa stained blood-films. Blood-derived MNC suspensions were obtained by Ficoll separation as described earlier [42]. Bone marrow cells were harvested by flushing the femur under sterile conditions with RPMI 1640 containing 500 µg/mL penicillin, 250 µg/mL streptomycin, and 2% fetal bovine serum (FBS, Gibco, Grand Island, NY). Single cell suspensions of spleen were prepared by mashing the organs and washing once in RPMI 1640 with 2% FBS. In transplantation experiments, 1.5×10^5 or 5×10^5 blood-derived MNCs from the donors were suspended in PBS containing 0.1% bovine serum albumin (BSA).

CFU-GM cultures

Cells were cultured as described previously [42]. Briefly, bone marrow cells were cultured in microtiter plates containing 10^4 cells per well in a semisolid methyl-cellulose medium in the presence of murine GM-CSF (1.25 ng/mL). Peripheral blood MNCs and spleen cells were cultured in 3.5 cm dishes containing 5×10^5 cells/mL and 10^6 cells/mL, respectively. After 6 days of culture, the numbers of CFU-GM, defined as aggregates of >20 cells, were scored using an inverted microscope.

Statistical analysis

Differences were evaluated using the Student *t*-test. In survival analysis, differences were evaluated using the Mantel-Haenszel test for linear association. *P*-values of <0.05 were considered statistically significant.

Results

Effects of single injections of cytokines on circulating progenitor cells

As reported previously, IL-8 induces a rapid mobilization of hematopoietic progenitor cells within 30 minutes after a single injection [32,33]. Since the effect of G-CSF or GM-CSF administered as single injections on mobilization of progenitor cells was unknown, we compared single injections of these cytokines with IL-8 with respect to their mobilizing capacity within 24 hours. Prior to the administration of mobilizing agents, the numbers of circulating CFU-GM were 28 ± 17 per mL blood (n=42). At 15–30 minutes after an injection of IL-8 (30 µg), numbers of circulating progenitor cells increased to 462 ± 335 CFU-GM/mL (n=40 for t=15, $p < 0.001$). A single injection of G-CSF or GM-CSF (2.5 µg), however, did not result in a significant increment in numbers of circulating CFU-GM up to 24 hours after injection (Fig. 1).

Effects of pretreatment with cytokines on the numbers of progenitor cells in the bone marrow and spleen

To increase the numbers of HPC in the bone marrow, mice were treated with either G-CSF, GM-CSF, IL-3, or SCF, 2.5 µg twice daily, for 2 or 4 days. Numbers of nucleated cells per femur increased significantly in mice treated with G-CSF for 2 days ($16.2 \pm 2.7\times10^6$ cells/femur, n=10; $p < 0.001$) or SCF for 4 days ($17.3 \pm 2.1\times10^6$ cells/femur, n=10; $p < 0.001$) compared with saline-treated controls ($10.1 \pm 4.6\times10^6$ cells/femur, n=42). Numbers of CFU-GM per 10^5 nucleated cells as well as numbers of CFU-GM per femur increased significantly in all mice pretreated with hematopoietic growth factors compared with saline controls (Table 1). Maximal increments were observed following treatment with G-CSF and SCF to 81,230 ± 16,424 and 74,916 ± 20,226 CFU-GM/femur, respectively, compared with saline controls (35,319 ± 15,367 CFU-GM/femur). In some experiments, the numbers of nucleated cells and CFU-GM per spleen were determined. As shown in Table 2, the cellularity of the spleen increased significantly after treatment with GM-CSF or IL-3 for 4 days. The numbers of CFU-GM increased 2- to 10-fold for all groups treated with HGF in comparison with saline controls.

Effect of splenectomy on IL-8-induced mobilization of CFU-GM

Pretreatment of mice with growth factors resulted in increased numbers of progenitor cells in the spleen. This was considered to be caused by homing of circulating progenitor cells in the spleen or by induction of progenitor cell proliferation in the spleen. Therefore, in some experiments, we studied the effects of splenectomy on the number of circulating progenitor cells. Prior to the administration of IL-8 to splenectomized mice, numbers of circulating leukocytes were significantly increased from $7.6 \pm 2.9 \times 10^6$ WBC per mL (n=42) in intact mice to $15.3 \pm 6.2 \times 10^6$ WBC per mL (n=33, $p < 0.001$), partially because of the increase in the numbers of circulating neutrophils from $1.4 \pm 0.6 \times 10^6$ per mL (n=42) to $2.8 \pm 2.1 \times 10^6$ per mL (n=33, $p < 0.001$; Table 3). The number of circulating progenitor cells was not increased in splenectomized mice (28 ± 17 CFU-GM per mL (n=42) vs. 25 ± 14 CFU-GM per mL (n=17) for intact mice ($p > 0.05$) (Table 3). At 15 minutes after the administration of IL-8, the increase in the number of circulating CFU-GM per mL blood was similar in splenectomized and intact mice [442 ± 300 CFU-GM per mL (range 238 to 808; n=12) and 462 ± 335 CFU-GM per mL (range 188 to 1172; n=42), respectively]. The kinetics of circulating CFU-GM were also similar in splenectomized mice and intact animals (Table 3). Based on these results, subsequent experiments were performed in intact mice.

Effect of pretreatment with growth factors on mobilization induced by IL-8

In control mice not treated with HGF, IL-8 induced a 17-fold significant increase in the number of circulating progenitor cells from 28 ± 17 CFU/mL (n=42) at t=0, to 462 ± 335 CFU/mL at 15 minutes after injection (mean ± SD, $p < 0.001$). To further increase the number of HPCs mobilized by IL-8, mice were pretreated with HGF before injection of IL-8 (Fig. 2). Pretreatment with IL-3 resulted in a 6-fold increase up to 368 ± 118 CFU-GM/mL (mean ± SD, n=4) in numbers of circulating progenitor cells after injection of IL-8 (t=0, 62 ± 52 CFU-GM/mL, mean ± SD, $p < 0.01$). G-CSF pretreatment resulted in an 8-fold increase from 314 ± 143 CFU-GM/mL at t=0 (n=4) to 2502 ± 513 CFU-GM/mL (n=4; mean ± SD, $p < 0.001$) at 15 minutes after injection of IL-8. After a 2-day treatment with GM-CSF, IL-8 induced a mean 2-fold increment from 168 ± 98 (n=7) to 402 ± 201 CFU-GM/mL (mean ± SD, n=7; $p < 0.05$). No effect of IL-8 on mobilization was observed after 4 days of treatment with GM-CSF (859 ± 387 to 1034 ± 421 CFU-GM/mL, mean ± SD, n=4 per group, $p > 0.05$). SCF pretreatment for 2 days did not by itself result in an increased number of circulating HPC. Following administration of IL-8 to mice pretreated with SCF, a similar fold increase in the number of progenitor cells was observed, compared with IL-8 mobilization without pretreatment, from 27 ± 15 to 524 ± 339 (mean ± SD, n=7 per group, $p < 0.005$).

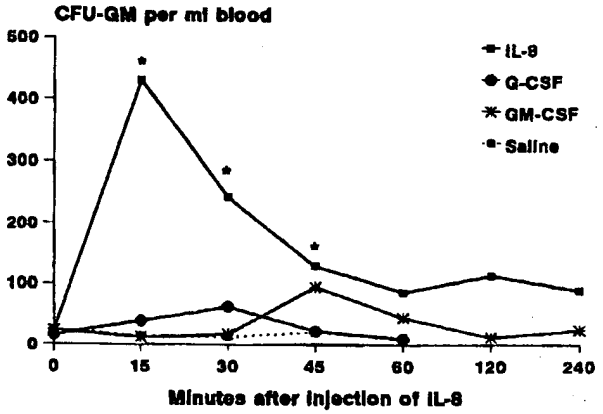


Fig. 1. Effect of a single ip injection of IL-8, G-CSF, and GM-CSF on the numbers of circulating CFU-GM. IL-8 was administered at a dose of 30 µg, G-CSF and GM-CSF at a dose of 2.5 µg in 0.1 mL PBS. Control mice received an equal volume of saline. Results are expressed as the mean of 4 to 42 mice per dose per time interval (seven experiments). *$p < 0.001$ compared with saline controls.

Radioprotective capacity of hematopoietic progenitor cells mobilized by IL-8

Since radioprotection may be a function of the numbers of transplanted progenitor cells, we calculated the mean number of CFU-GM transplanted following treatment with various growth factors. As shown in Figure 3, there was a tendency (correlation coefficient, 0.76) of improved radioprotection following transplantation of higher numbers of CFU-GM. To study the radioprotective capacity of the mobilized progenitor cells, recipient female mice were lethally irradiated

Table 1. Numbers of hematopoietic progenitor cells in bone marrow after pretreatment with growth factors

Pretreatment	Bone marrow		
	NC/femur ($\times 10^6$)	CFU-GM/10^5 NC	CFU-GM/femur
Saline	11.0 ± 5.0	331 ± 63	35,319 ± 15,367
G-CSF 2 days	16.2 ± 2.7**	500 ± 76**	81,230 ± 16,424**
GM-CSF 2 days	10.0 ± 0.8	394 ± 57*	39,221 ± 5,208*
GM-CSF 4 days	9.4 ± 1.2	445 ± 57**	41,655 ± 6,460*
IL-3 4 days	7.8 ± 2.0	602 ± 44**	46,199 ± 9,306**
SCF 4 days	17.3 ± 2.1**	430 ± 62**	74,916 ± 20,226**

Balb/c mice were injected ip with 2.5 µg growth factor or saline twice daily. Data represent the mean ± SD of two to five experiments (n=42 for saline, 10 for G-CSF, SCF, and GM-CSF 4 days, 20 for GM-CSF 2 days and IL-3. *$p < 0.05$, **$p < 0.001$ compared with saline controls.

Table 2. Numbers of hematopoietic progenitor cells in the spleen after pretreatment with growth factors

Pretreatment	Spleen		
	NC/spleen ($\times 10^6$)	CFU-GM/10^5 NC	CFU-GM/spleen
Saline	127 ± 27	41 ± 27	4,969 ± 2,393
GM-CSF 2 days	142 ± 24	87 ± 24*	12,592 ± 5,037*
GM-CSF 4 days	216 ± 41*	232 ± 59*	50,617 ± 18,756*
IL-3 4 days	191 ± 34*	133 ± 43*	25,833 ± 9,787*

Balb/c mice were injected ip with 2.5 μg growth factor or saline twice daily. Data represent the mean ± SD of two or five experiments (n=42 for saline and 10 for the growth factors).
*$p < 0.001$ compared with saline controls.

(8.5 Gy), and transplanted with 1.5 or 5×10^5 blood-derived mononuclear cells obtained from male donors at 15 minutes after an ip injection of saline or IL-8 (30 μg per mouse). Cell doses were chosen to result in suboptimal survival rates of approximately 50 or 70% for recipients receiving PBMNCs from IL-8-primed donors [32]. The radioprotection rate of lethally irradiated recipients was increased by transplantation of PBMNC of donors pretreated with IL-3 (16% survival, three experiments n=20; Fig. 4C), GM-CSF (20% survival, two experiments, n=20; Fig. 4B), SCF (40% survival, two experiments, n=20; Fig. 4A), and G-CSF alone (80% survival, two experiments, n=15; Fig. 4B), compared with saline controls. Transplantation of circulating progenitor cells mobilized by IL-8 after pretreatment with SCF resulted in radioprotection of all recipients, which was significantly better than controls transplanted with cells obtained after treatment with either factor alone. In contrast, transplantation of blood-derived blood cells mobilized by IL-8 following pretreatment with GM-CSF, G-CSF, or IL-3 did not enhance radioprotection compared with similar numbers of PBMNCs derived from donors treated with IL-8 alone (Fig. 4).

Discussion

We have shown previously that a single injection of IL-8 is capable of mobilizing progenitor cells [32,33]. In this report, we describe the effects of pretreatment of donor-mice with various growth factors in an attempt to enhance IL-8-induced mobilization of hematopoietic progenitor cells. To study whether these cytokines, at the doses and schedule used, were able to interfere with the rapid IL-8-induced mobilization, we first studied whether single bolus injections of the most commonly used mobilizing agents, G-CSF and GM-CSF, were able to mobilize HPC in an equally rapid fashion. But no such effect was observed. Accordingly, Gasparetto et al. reported no consistent differences in progenitor cell concentrations after a single bolus injection of recombinant human (rh)-G-CSF, rh-GM-CSF, or rh-PIXY 321 in rhesus monkeys [43]. Mobilization after a single bolus injection of an agent is described previously for IL-1, anti-VLA_4-antibodies, endotoxin, and complement only [42–46]. Complement-dependent mobilization showed a response as rapid as IL-8, whereas IL-1 and anti-VLA_4-antibodies increased numbers of circulating progenitor cells after 4 and 24 hours, respectively.

In mice pretreated with SCF for 2 days, similar numbers of circulating CFU-GM were observed compared with animals treated with IL-8 alone. Mice pretreated with IL-3, G-CSF, or GM-CSF showed increased numbers of circulating progenitor cells compared with animals treated with IL-8 alone, although the fold increment decreased, indicating an additive effect rather than a synergistic one. After the additional bolus-injection of IL-8, their numbers further increased, but no enhanced radioprotection was observed following transplantation of equal numbers of mobilized cells obtained from these animals. These data indicate that, despite increased numbers of HPC, no significant enhancement in the number of cells responsible for radioprotection occurred. In contrast, SCF treatment for 2 to 4 days did not by itself induce mobilization of committed progenitor cells, and IL-8-induced

Table 3. Number of leukocytes and hematopoietic progenitor cells in the peripheral blood in intact and splenectomized mice after injection of IL-8

Minutes after IL-8 injection	Mice	WBC$\times10^6$/mL	PMN$\times10^6$/mL	CFU-GM/mL
0	Intact	7.6 ± 2.9	1.4 ± 0.6	28 ± 17
0	Splenectomized	15.3 ± 6.2**	2.8 ± 2.1**	25 ± 14
15	Intact	8.0 ± 3.2	0.6 ± 0.6	462 ± 335
15	Splenectomized	12.8 ± 7.3*	1.2 ± 2.0	442 ± 300
30	Intact	7.4 ± 3.8	0.9 ± 1.4	334 ± 196
30	Splenectomized	13.1 ± 4.4*	2.6 ± 2.2*	397 ± 235
45	Intact	6.4 ± 4.1	1.0 ± 1.8	120 ± 65
45	Splenectomized	10.3 ± 5.3*	2.6 ± 3.1	99 ± 101

Intact or splenectomized Balb/c mice were injected ip with 30 μg IL-8 at t=0. Data represent the mean ± SD of two or 4–7 experiments (n=12–42 for intact mice, 4–17 for splenectomized mice).
*$p < 0.01$ and **$p < 0.001$ compared with intact mice.

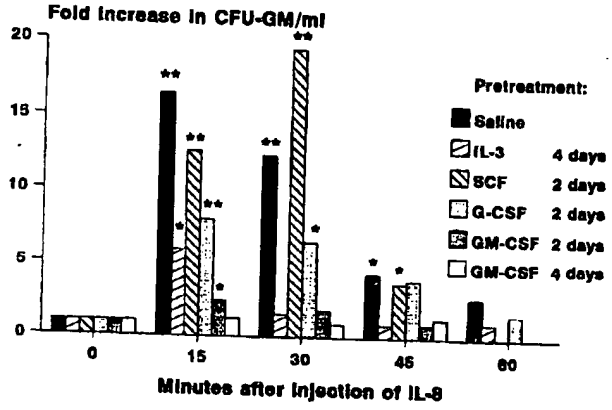


Fig. 2. Increment in numbers of circulating CFU-GM per mL blood after a bolus injection of IL-8 in mice pretreated with various cytokines. Results are expressed as fold increments in comparison with progenitor cell numbers prior to IL-8 injection. Numbers of circulating CFU-GM per mL of blood prior to injection of IL-8 after pretreatment were 28 ± 17 (Saline), 68 ± 52 (IL-3), 27 ± 15 (SCF), 314 ± 143 (G-CSF), 168 ± 98 (GM-CSF for 2 days), and 859 ± 387 (GM-CSF for 4 days). Data are expressed as the mean ± SD of 4 to 42 mice per dose per time interval (two to seven experiments per group).
*$p < 0.05$, **$p < 0.005$.

mobilization in animals pretreated with SCF was equally effective as in animals pretreated with saline. Transplantation of equal numbers of cells derived from donors pretreated with SCF and mobilized with IL-8 resulted in 100% radioprotection, however, which was significantly better than that obtained in controls transplanted with cells mobilized by IL-8

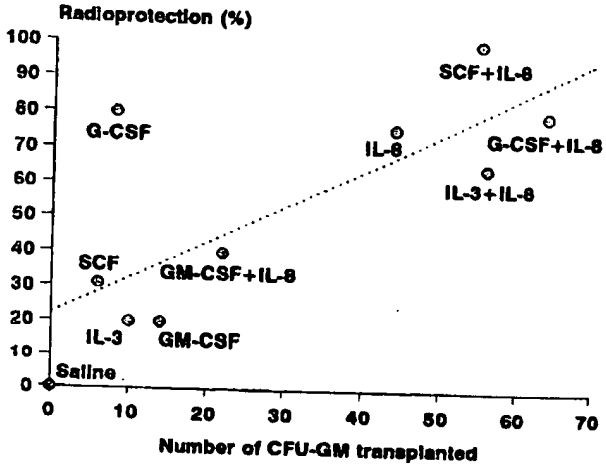
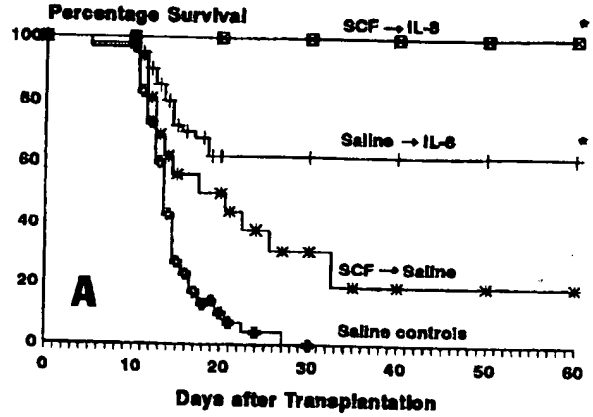
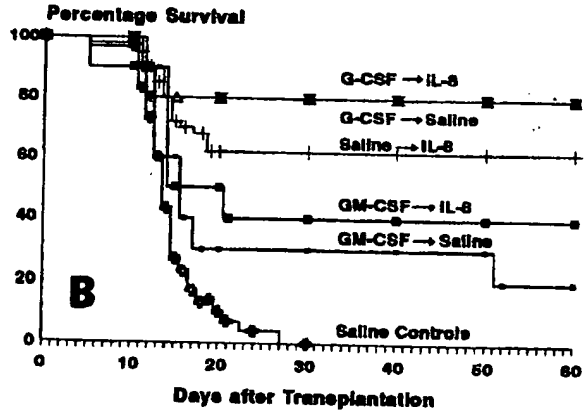


Fig. 3. Correlation between numbers of CFU-GM transplanted into lethally irradiated mice and survival of the recipients. Mobilizing cytokines used are plotted next to the dots. The dashed line represents the trend (correlation coefficient 0.76).





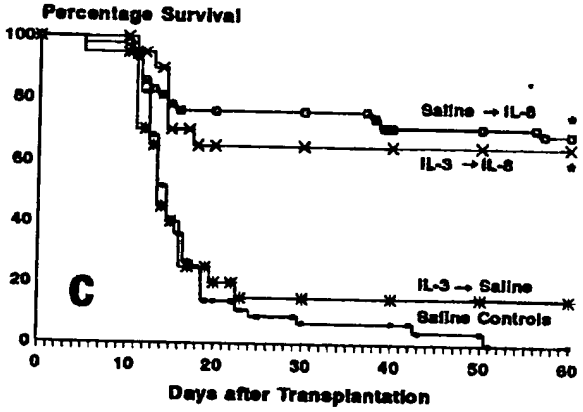


Fig. 4. Survival of lethally irradiated recipients at 30 days after transplantation of 1.5×10^5 (A,B) or 5×10^5 (C) PBMNCs per animal. PBMNCs were derived from donor mice pretreated with SCF (A), G-CSF or GM-CSF (B), or IL-3 (C) at a dose of 2.5 μg twice a day for 2 or 4 days prior to a single bolus injection of 30 μg IL-8 or saline. Results are expressed as the mean of 10 to 42 animals in 2–7 experiments per group.
*$p < 0.01$ compared with animals receiving a bolus injection of saline after pretreatment.

alone. These results indicate that mobilization of CFU-GM may or may not coincide with mobilization of cells exhibiting radioprotection and therefore gives no clue as to the role of CFU-GM in radioprotection. Maximum numbers of circulating CFU-GM were observed in mice treated with G-CSF and GM-CSF for several days, emphasizing the potency of these factors in mobilization of CFU-GM. Under these circumstances, no further increase in circulating progenitor cell numbers was observed after injection of IL-8, possibly indicating that maximum mobilization was already induced by growth factor treatment alone.

In some other in vivo studies, SCF was successfully tested in combination with G-CSF to increase numbers of circulating stem cells in mice, monkeys, and dogs [34–37]. In these studies, SCF significantly increased the yield of G-CSF-induced mobilization of long term reconstituting progenitor cells [36–39]. We therefore conclude that SCF-pretreatment of donors does not improve the mobilization of the more committed progenitor CFU-GM compared with or in combination with IL-8, but does greatly improve the mobilization of cells exhibiting radioprotection compared with IL-8 as a single factor. The results presented here suggest that pretreatment of the donor with SCF prior to a bolus-injection of IL-8 may be useful to increase mobilization of cells exhibiting radioprotection, resulting in improved survival of recipients.

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